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Regulation of the Transcription and Replication Cycle of Human Cytomegalovirus Is Insensitive to Genetic Elimination of the Cognate NF-κB Binding Sites in the Enhancer

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The role of NF-κB in regulating human cytomegalovirus (HCMV) replication and gene transcription remains controversial. Multiple, functional NF-κB response elements exist in the major immediate-early promoter (MIEP) enhancer of HCMV, suggesting a possible requirement for this transcription factor in lytic viral replication. Here we demonstrate by generating and analyzing HCMVs with alterations in the MIEP-enhancer that, although this region is essential for HCMV growth, none of the four NF-κB response elements contained within the enhancer are required for MIE gene expression or HCMV replication in multiple cell types. These data reveal the robustness of the regulatory network controlling the MIEP enhancer.

The major immediate-early promoter (MIEP) of human cytomegalovirus (HCMV) is responsive to a multitude of transcription factors and plays a pivotal role in initiating the viral transcription/replication cycle (7, 16; reviewed in references 22 and 23). Regulation of the MIEP has been postulated to be critical in determining HCMV permissiveness and the transition between latent and lytic infection. Thus, deciphering the molecular mechanisms of the MIEP regulation may reveal key control points contributing to HCMV pathogenesis.

The MIEP enhancer includes four cognate NF-κB recognition sites, and NF-κB activates MIEP transcription in transient-transfection assays (20, 25–27). HCMV infection results in rapid induction of cellular NF-κB (19, 27, 30), and several groups have reported a potential contribution of NF-κB to the replication strategy of HCMV through regulation of the MIEP (8, 13). In contrast, we and others have reported a neutral or even a negative role of NF-κB activation on HCMV transcription/replication cycle in different cell types (3, 4, 11, 14, 15). However, the basis for these experimental discrepancies is currently unclear. Importantly, a direct test of the requirement for the MIEP NF-κB binding sites in HCMV transcription/replication has still not been performed. Here we report on formally assessing the direct requirement of the cognate binding sites for NF-κB in contributing to major immediate-early (MIE) transcription and viral growth.

As a first step toward understanding NF-κB regulation of the HCMV MIEP, we deleted enhancer sequences from −52 to −667 (including all NF-κB response elements), in HCMV AD169. A parental HCMV bacterial artificial chromosome (BAC) (5, 6) containing the E-GFP open reading frame (ORF) under control of the murine cytomegalovirus (MCMV) MIEP (Fig. 1A, line 1) was used to construct two enhancerless HCMV recombinant mutants. In HCMVΔE::Kan, MIEP sequences from −52 to −667 were removed (Fig. 1A, line 2), and in HCMVΔE::Kan, enhancer sequences were replaced with a 1-kbp stuffer region to maintain the genomic spatial integrity of the ie1/ie2 and UL127 promoters (Fig. 1A, line 3). Once the integrity of constructed HCMV genomes was confirmed by restriction analysis (data not shown), they were transfected in MRC-5 fibroblasts. Three days posttransfection, ∼100 single cells expressing green fluorescent protein (GFP) could be detected in all cultures (see Fig. 1B, panels D, G, and J). Cells transfected with the parental HCMV BAC yielded viral plaques (Fig. 1B, panel E) that progressed to complete cytopathicity (panel F), whereas cultures transfected with the enhancerless HCMV BACs did not result in viral spread (panels H, I, K, and L). These data indicate that deletion of the entire MIEP enhancer region of HCMV genome is lethal.

To verify that the generated HCMV mutants were defective due to deletion of the enhancer, the ability of an IE1/IE2 expression plasmid (pSVH) (29) to rescue replication was tested. Cotransfection of enhancerless BACs with pSVH resulted in spread of GFP-expressing virus to adjacent cells (Fig. 1B, panels M to O and P to R), ultimately leading to a complete cytopathic effect. In addition, a revertant HCMVΔE virus was generated by the ET BAC mutagenesis method (5, 24) and shown to replicate with identical kinetics to the parental HCMV in MRC-5 cells (Fig. 1C). Thus, these results indicate that deleting the entire MIEP enhancer in HCMV AD169 abolishes lytic viral replication in cultured fibroblasts and are consistent with previous results resecting MIEP enhancer sequences in the Towne strain of HCMV (18, 21). Here, through the rescue experiments, we have eliminated the possibility that the replication defects seen in the enhancerless HCMV recombinants were due at least in part to alterations in other regions.

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of their genome. These observations are also in line with the absolute requirement of the enhancer during the acute MCMV infection (17).

To directly analyze the role of NF-κB in regulating the MIEP, we specifically disrupted the four enhancer NF-κB binding sites. The point mutations introduced in the MIEP (3) are illustrated in Fig. 2A. It should be noted that these four sites are the only NF-κB binding sites present in the MIE enhancer and that, in the context of the whole genome, NF-κB recognition sites have only been described to date in another location, the US3 immediately-early (IE) enhancer (9). Transfection of human U937 cells with reporter plasmids containing
either the wild-type or the NF-κB mutant MIEP revealed that tumor necrosis factor alpha (TNF-α)-induced MIEP activity was abolished in the mutant, whereas basal transcription was not affected (Fig. 2B). These results are consistent with previous studies indicating a role for NF-κB in regulating MIEP activity in transfection-based assays. To then test whether the four cognate NF-κB response elements in the MIEP are required for HCMV replication, two independent HCMV recombinants mutated in the NF-κB binding sites were generated (HCMV.NFkBα and HCMV.NFkBβ). HCMV.NFkBα was constructed by using the ET mutagenesis method and subsequent transfection of the mutant BAC in MRC-5 cells, whereas HCMV.NFkBβ was generated by cotransfecting MRC-5 cells with HCMV.dE, pSVH, and pUChEnh.NFkB, a plasmid that carries HCMV sequences from nucleotide 171443 to 176844 (10) in which the four NF-κB elements of the enhancer were mutated. Infectious recombinant viruses were recovered from the transfections, used to infect new cell monolayers, and plaque purified three times. Viral stocks of both HCMV.NFkBα and HCMV.NFkBβ were prepared, their genomic integrity was verified by restriction digestion (data not shown), and the successful disruption of the enhancer NF-κB binding sites was confirmed by PCR analysis (Fig. 2C) and the nucleotide sequence of the MIEP region (data not shown).

We next assessed the effect of abrogating the enhancer NF-κB recognition sites on expression emanating from the MIEP in the context of the infection of human embryonic lung (HEL) fibroblasts with either wild-type or mutant viruses. As shown by real-time PCR, IE1 mRNA levels were comparable or even slightly higher (48 h postinfection [hpi]) in cells infected with HCMV.NFkBα and HCMV.NFkBβ than in parental HCMV-infected cells (Fig. 3A). In addition, HEL cells were infected throughout a 72-h period with the three viruses and subjected to Western blots by using a monoclonal antibody specific for the IE1 protein. We could not detect significant differences in the expression of the IE1 protein between HCMV and the HCMV.NFkB mutants at any of the time points analyzed (Fig. 3B). Furthermore, treatment of cells with the NF-κB inducer TNF-α (Fig. 3B) did not result in differential expression of IE1 in cells infected with wild-type or mutant viruses. Consequently, we conclude that the NF-κB binding sites in the MIEP do not significantly influence IE1 gene transcription or expression in lytically infected fibroblasts.

To examine the requirement for the enhancer NF-κB binding elements on HCMV growth, kinetic studies with HCMV, HCMV.NFkBα, or HCMV.NFkBβ were performed on HEL fibroblasts. No difference in viral production at any time point was observed between the mutants and the parental virus (Fig. 4A). Treatment of cultures with TNF-α drastically inhibited HCMV growth, likely due to the induction of beta interferon (4; data not shown). In order to examine the replication capacity of HCMV.NFkBα mutants in other cell types, we tested lung fibroblasts (MRC-5), U373 MG cells derived from glioblastoma, retinal pigment epithelium (RPE) cells, and differentiated embryonal carcinoma cells NTera2 (NT2/D1). Importantly, parental and mutant viruses replicated in a comparable manner (Fig. 4B to E), strongly suggesting a neutral role of NF-κB for HCMV acute transcription/replication cycle in different cell types in culture.

The molecular details on the activation of the MIEP during HCMV infection are still poorly understood. In the present study, we demonstrate by generating and characterizing HCMV recombinants with alterations in the MIEP enhancer that (i) the enhancer region is necessary for HCMV growth
FIG. 3. Analysis of ie1 gene expression in HCMV.NFkB-infected cells. (A) Real-time PCR analysis of ie1 RNA expression in cells infected with HCMV.NFkB. HEL fibroblasts were infected at an MOI of 0.01 with HCMV, HCMV.NFkBa, or HCMV.NFkBb and harvested at the time points after infection indicated for isolation of RNA and subsequent analysis by real-time PCR using primers within exon 4 of the HCMV ie1 gene as previously described (3). The results are presented as the relative amount of ie1 mRNA normalized to 18S rRNA, and error bars represent the standard errors of the means. (B) Expression kinetics of the IE1 protein by HCMV.NFkB mutants. HEL fibroblasts were mock-infected or infected at an MOI of 0.6 (for the 4-, 6-, and 12-h time points) or 0.1 (for the 24, 48, and 72 h time points). Where indicated, cells were treated with 10 ng of TNF-α/ml 2 h before infection, during, and immediately after the adsorption period. At the indicated time (in hours) postinfection (hpi), samples were lysed, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 7% gels, transferred to nitrocellulose as previously described (1), and probed with an HCMV IE1 specific monoclonal antibody (MAB810; Chemicon, Temecula, CA). As an internal control, actin immunodetection was performed with a monoclonal antibody (A2066; Sigma, St. Louis, MO).

FIG. 4. Growth kinetics of HCMV.NFkB mutants. HEL (A), MRC-5 (B), RPE (C), U373 (D), and NT2/D1 cells (E; differentiated for 5 days with 10−5 M retinoic acid) were infected at an MOI of 0.025 (HEL and MRC-5) or 1 (RPE, U373, and NT2/D1, resulting in ca. 10 to 25% of GFP-positive cells in the culture at 48 hpi) with HCMV, HCMV.NFkBa, or HCMV.NFkBb. At the indicated time in days postinfection, the amount of extracellular (HEL, MRC-5, and RPE) or cell associated (U373 and NT2/D1) infectious virus present in the cultures was determined by plaque titration assays on MRC-5 cells. Each datum point represents the average and standard deviation from three separate cultures. Dashed lines represent the limits of detection.
and (ii) the MIEP NF-κB response elements do not contribute to lytic replication of HCMV in multiple cell types.

The involvement of NF-κB activity on MIE expression and HCMV replication has been a controversial issue (3, 4, 8, 11–15). The discrepancy pivots around whether NF-κB directly or indirectly influences HCMV transcription and/or replication. It is generally assumed in the field that the activation of NF-κB results in the direct stimulation of the MIEP and, as a consequence, in increased, i.e., gene expression and viral replication. Our observations are in agreement with the fact that NF-κB contributes to HCMV MIEP-enhancer activation in transient-transfection assays, as has been documented in a number of reports. However, we clearly show here that the cognate NF-κB binding sites within the enhancer do not play a major independent role in the transcription/replication strategies of HCMV in a variety of cell types. We demonstrate in a direct manner that, in the context of the infection, the enhancer is insensitive to mutations in the NF-κB binding sites, underlining a high level of robustness of the associated regulatory network controlling this region. While these results are in line with previous observations (3, 11, 14, 15), they are in contrast with others (8, 12, 13). In these apparently contradictory studies, a positive involvement of NF-κB in MIEP transcription and HCMV replication has been observed by blocking NF-κB activity using a variety of pharmacologic agents exhibiting a range of specificity and selectivity. These studies provide an indirect test and, moreover, the selectivity of the agents (e.g., aspirin or MG-132) used to inhibit NF-κB signaling pathways should be taken in consideration. It must be also noted that although to date only NF-κB sites have been found in the MIEP and in the US3 IE enhancer (9), the presence of additional NF-κB responsive genes in the HCMV genome could account in part for some of the discrepancies found in different studies.

Our data do not exclude the possibility that NF-κB may regulate HCMV MIE gene expression or growth in cell types not examined here or are required for in vivo replication and/or reactivation from latency. The fact that the viruses used in the present study derive from the HCMV laboratory strain AD169 prevented their analysis in other cell types more relevant for HCMV infection, including macrophages, endothelial, or dendritic cells. Mouse CMV recombinants containing the HCMV MIEP (with or without mutations in the NF-κB response elements) replicate and establish latency in the mouse (1, 3; A. Angulo unpublished results), permitting us to explore these aspects in the context of an in vivo infection.

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